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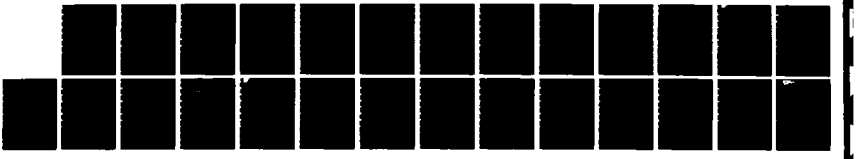

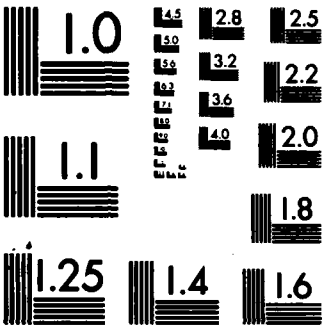


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# Isolation and Characterization of Erythrocyte and Parasite Membranes from Rhesus Red Cells infected with P. Knowlesi

Annual Report

Donald F. Wallach, M.D.

June 1981

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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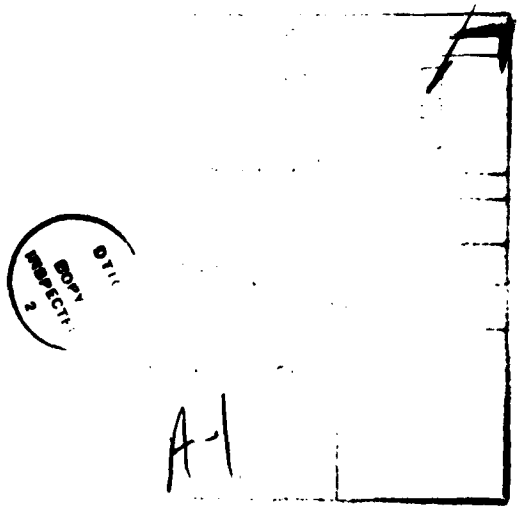
FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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# ANNUAL PROGRESS REPORT

Donald F. H. Wallach, M. D.

Contract No. DAMD 17-74-C-4118

Enclosure 1

ANNUAL PROGRESS REPORT

## 1. Membrane fractionation

### 1.1. Subfractionation of monkey erythrocytes parasitized by P. knowlesi

Purified schizont (6 - 10 nuclei) and erythrocyte membranes from parasitized cells have been isolated according to techniques developed in our laboratory (Wallach and Conley, 1977). The purity of the fractions obtained has now been further tested by high-resolution, high-specificity protein fractionation techniques, as detailed in section 2.

### 1.2. Subfractionation of the intracellular parasite

Each intact schizont released from infected erythrocytes by controlled nitrogen decompression (1) is surrounded by two membranes, its own plasma membrane and the membrane of the parasitophorous vacuole. We have initiated experiments to separate and isolate the vacuolar membrane and the parasite plasma membrane. For this, the surfaces of intact schizonts, released from surface-iodinated infected erythrocytes (1), was surface-labeled with $^{125}I$, using lactoperoxidase-catalyzed radioiodination essentially as in (1). The parasites were then disrupted by nitrogen decompression (450 psi for 20 min; $2 \cdot 10^7$ g · min per ml $NaCl/PO_4$, 0.13 M, 0.02 M, pH 7.5). This procedure yielded vesicles originating from both the vacuolar, and parasites' surface membranes as discussed by us for cells of other types (2); at the same time, most of the mitochondria and nuclei of the parasite stayed intact. The homogenate was centrifuged at $10^7$ g · min, resuspended in 25% of the original volume and loaded atop a dextran step gradient as described in Fig. 1.

Equilibrium ultracentrifugation at $\sim 10^7$ g · min results in six fractions (Fig. 1A-C). Of these the low density fractions 1, 2 and 3 (Fig. 1A-C) consist of soluble proteins (fraction 1), and small membrane vesicles whereas undisrupted parasites, nuclei and mitochondria concentrate at higher densities (Fractions 4-6). In gradients A and B (Fig. 1) we compare disrupted parasites from iodinated erythrocytes and parasites surface-iodinated by

a second $^{125}$I-labeling step before disruption. About 70-75% additional $^{125}$I is introduced into the isolated parasites after release from surface-iodinated erythrocytes (Fig. 1A,B). Of this activity, 65 - 70% is recovered in the low density fractions 1 - 3. These fractions therefore, contain 35% of the total $^{125}$I-radioactivity after the second iodination in comparison to only 15% in gradient A. If the isolated parasite is labeled a second time but fractionated without disruption only 13% of the $^{125}$I-activity is recovered in fractions 1 - 3, and most of the activity concentrates in the high density fractions at the density of the parasite itself (density 1.10 - 1.12).

Preliminary comparative analyses of the protein composition of washed, Triton X-100 solubilized membrane vesicles from fraction 2, 3 and 4 using isoelectric focusing in polyacrylamide (see section 3.2.) reveal clear differences, which are yet to be defined in detail.

1.3. Conclusions.

Extension of the fractionation approach given above will allow us to identify and characterize proteins typically associated with the parasite surface membrane and other subcellular organelles. Furthermore, we shall be able to eliminate and isolate erythrocyte membrane contaminants or vesicles originating from the vacuolar membrane by employing affinity density perturbation. In this technique immunoglobulin against normal erythrocyte membrane proteins (see section 2.1.1.3.) will be coupled to latex beads which will then be reacted with the parasite homogenate. This will allow us to selectively increase the density of membrane vesicles containing proteins characteristic of normal erythrocyte membranes and to isolate those membranes by centrifugation in a density gradient (2).

2. Membrane protein analyses

2.1. Immunochemical techniques

2.1.1. Antisera

2.1.1.1. Immunization of rhesus monkey with purified P. knowlesi

P. knowlesi schizonts, isolated as in (1), were resuspended in 0.25 M

sucrose, 10 mM HEPES, pH 7.5 ($10^8$ parasites/0.25 ml), mixed with an equal volume of complete Freund's adjuvant (CFA) and injected subcutaneously into a rhesus monkey at multiple sites at the back (to prevent major necrotic skin lesions). Monthly injections over a period of one year yield a potent humoral immune response in the animal (see section 2.1.3.). All immunochemical analyses (Fig. 2-4) were performed using the monkey anti-parasite (MαP) serum drawn after the eighth booster. If CFA is replaced by incomplete Freund's adjuvant (IFA) the titer of the serum is substantially reduced. The titer can be raised again when IFA is replaced by CFA. This indicates that CFA is essential for the immunization scheme employed.

2.1.1.2. Conclusions

Our current immunization scheme using purified intracellular P. knowlesi parasites in CFA allows us to produce a high titered anti-serum in the natural host of this parasite. The serum strongly agglutinates isolated parasites and infected erythrocytes (SICA) and is also suitable for immunochemical analyses.

2.1.1.3. Rabbit antisera against normal-monkey erythrocyte membranes (RαNMEM).

Rabbits are initially injected s.c. with 0.5 mg of NMEM in CFA (v/v 1/1; 1 ml total volume). The animals are then boostered with 1 mg of membrane protein mixed with CFA by multiple s. c. injections at monthly intervals. After two boosters an antiserum suitable for immunochemical analyses (see Fig. 7) was obtained. Sera are always drawn ten days after the booster.

2.1.1.4 . Processing, storage

All antisera are processed sterilely and stored at -70°C in small aliquots.

2.1.2. Methods

Crossed immune electrophoresis (CIE). We use the guidelines and cast 1% agarose containing 1% Triton X-100 onto 50 x 50 mm glass plates to produce layers 1.5 mm thick. The buffer is 0.038 M Tris/0.1 M glycine, pH 8.7. Triton X-100 solubilized membrane protein (150 µg) is electrophoretically separated for 60 minutes with a voltage of 10 V/cm of separation distance.

1 cm agarose strip containing the separated proteins is left on the plate and the agarose remaining on the plate is then replaced by agarose containing 0.04 ml antiserum /ml and 1% Triton X-100. Using 7V/4 cm (monitored on the agarose plate), we electrophorese the preseparated membrane proteins at right angles to the first dimension into the antibody-containing agarose. The electrophoresis is done at pH 8.7 to prevent electrophoretic migration of antibodies. The washed, dry plates are stained with Coomassie blue as described in (4).

Crossed-line immune electrophoresis (CLIE) In this form of CIE designed to determine cross-reactivities, an agarose strip containing the protein(s) in question is cast between the strip bearing the electrophoretically separated membrane proteins and the antibody-containing agarose. Cross-reactivity is indicated when the origins of the immunoprecipitation arcs are anodally displaced after electrophoresis in the second dimension. (3).

2.1.3. Results

The immune precipitation pattern of isolated P. knowlesi schizonts and membrane purified from infected erythrocytes electrophoresed against monkey anti-parasite serum is shown in Figs. 2 and 3 A, B, respectively.

As shown in Fig. 2, infected membranes reveal seven, in part complex precipitation arcs, numbered 1 - 7 according to electrophoretic mobility in the first dimension. Of these antigens only weakly reacting components 1 and 3 and the strong component 7 are also found in the parasite used as antigen for immunization (Fig. 3 A, B).

The identity of components 1, 3 and 7 in parasites and infected membranes is documented by CLIE, as shown in Fig. 4 B. Precipitate 7 (7i; from infected membrane) is anodally displaced and continues into the precipitation line 7p of the parasite material polymerized into the intermediate strip (I) (see Fig. 4 A for comparison). Similarly, precipitates 1i and 3i originate from precipitation lines 1p and 3p (not marked), respectively. There is no

immunological cross-reactivity between any protein in membranes from normal monkey erythrocytes and antigens 1 - 7 of infected membranes (Fig. 4 A). These data are further supported by the absence of immune precipitates in CIE when membrane proteins from normal erythrocytes are electrophoresed against monkey anti-parasite serum (data not shown).

2.1.4. Discussion

The different relative concentrations of components 1 and 7 in membranes of infected cells and parasites argue against simple contamination of the former (ratio of the heights of precipitate 1:7 is about ten times higher in "infected membranes" than in the parasite). Rather the results suggest that the intracellular parasite and the membrane of parasitized erythrocytes have antigens in common. Equally or more intriguing is the finding that the "infected membranes" reveal a greater number of parasite-specific antigens than the parasite used for immunization.*

We interpret our immunochemical results as follows:

Successful intraerythrocytic maturation demands protein functions of the host-cell membrane — the primary extracellular/intracellular boundary — that the normal erythrocyte membrane does not possess (e.g. transport carriers). The required proteins are synthesized by the parasite and inserted into the host-cell membrane. Then they are detected as SICA antigens, by immunoelectronmicroscopy (5) or by our immunological methods as immune precipitates. That they are not detected in the parasites may signify that they are not retained in sufficient amounts there to produce immune precipitates visible using our present methods. It is also possible that the antigens are shielded in the parasite and become expressed when bound to the host-cell membrane or when injected for immunization.

These immunochemical results are in preparation for publication (Schmidt-

* Because parasite preparations from four different monkeys with different blood groups have been used for immunization the possibility of antibodies against trace contaminants in normal erythrocytes is highly unlikely. This is further supported by data described in section 2.3.2.

Ullrich and Wallach).

## 2.2. Isoelectric focusing (IEF)

This high-resolution technique has great value by itself but proves most useful in bidimensional approaches, i. e. isoelectric focusing linked to dodecyl sulfate polyacrylamide gel electrophoresis and immune electrophoresis, respectively (see section 2.3.). We have applied focusing approaches to better define the immunochemical phenomena already described.

Fig. 5 shows the standard protein pattern obtained when Triton X-100 solubilized membrane proteins from normal and parasitized erythrocytes and proteins of isolated parasite are fractionated by isoelectric focusing. In all cases 100 - 200 µg of protein is focused in 4% polyacrylamide containing 2% ampholytes (Ampholine pH 3.5 - 10.0), 8 M urea and 1% Triton X-100 as described by us (6). Major differences are noted when normal and "infected" membranes on the one hand and "infected" membrane and parasite on the other are compared. Membranes from infected cells show new components at pI 4.2, 4.3, 4.6 and 4.7 which are not found in normal membranes but do occur in the parasite. On the other hand, some major components of normal membranes, with pIs above pH 6.5 are almost deleted in "infected" membranes; they probably represent spectrin.

## 2.3. Bidimensional techniques

### 2.3.1. Bidimensional isoelectric focusing - dodecyl sulfate polyacrylamide gel electrophoresis (IEF-DS-PAGE).

Isoelectric focusing is in cylindrical polyacrylamide gels (65 mm x 3 mm; ∿ 350 µg of protein per gel; composition as in section (2.2.) with a pH 3.5 - 10 gradient. The catholytes and anolytes are 0.03 M NaOH and 0.05 M $H_2SO_4$ respectively. For SDS-PAGE in the second dimension, we use gel slabs (75 x 75 x 2.75 mm). Prior to the second-dimension step, the slabs are pre-electrophoresed with 0.04 M Tris/0.02 M acetate/2 mM EDTA/1% DS (wt/vol), pH 7.4 for 15 min at 25 mA per slab. At the same time, the focusing gels are equilibrated with DS/electrophoresis buffer (3% SDS and 0.12 M dithiothreitol), 10 ml of buffer per gel and buffer changed every

10 min for 50 min. These washes introduce the detergent and reducing agent required for the second-dimension separation and also eliminate the focusing pH gradient. The focusing gel is then positioned on top of the pre-electrophoresed gel slab and electrophoresis is for 16 hr at 8 mA per slab as described in ref. 6.

IEF-SDS-PAGE of membrane proteins from normal and parasitized erythrocytes and purified parasites (Fig. 6, A-C) reveals at least 59 individual protein spots for each of the three fractions analysed. There are no components common to normal erythrocyte membranes and purified parasites excluding contamination of parasite by host erythrocyte membrane. The data indicate that the surface membrane of parasitized monkey erythrocytes undergoes substantial modifications. Several major membrane components, i. e. spectrin and parts of the "band 3" complex, as well as some smaller mol. wt. components, are deleted or significantly diminished (Fig. 6 D). The membranes from infected cells also contain some small amounts of parasite protein, but the different proportions of these components in the parasite and the membranes of infected cells indicate mechanisms other than simple contamination. Proteins qualitatively common to parasite and membranes of parasitized cells are depicted in Fig. 6 E. As a third group of alterations in the membrane of the parasitized erythrocyte are discrete modification of some proteins in terms of electric points or molecular weights. These minor modifications of the proteins are marked by arrows in Fig. 6 B.

2.3.2. Bidimensional isoelectric focusing-immune electrophoresis (IEF-IE)

The procedure is as described in (6). Isoelectric focusing is in gel slabs (4% acrylamide, crosslinked with 2.5% bisacrylamide) containing 2% ampholytes (pH 3.5 - 10), 8 M urea, 1% Triton X-100, and 10% sucrose. The catholyte is 1 M NaOH and the anolyte 1M $H_3PO_4$. We apply about 200 µg of protein per lane. After focusing the 10 x 90 mm polyacrylamide section containing the focused proteins is sliced into two 5 x 90 mm strips. One of these is washed three times (10 min each) in 20 ml of 0.038 M Tris/0.1 M glycine, pH 8.7/1% Triton X-100 and then immunoelectrophoresed. The other strip is stained as described in (6).

For the second dimension immune electrophoresis, we cast 80 x 80 mm immunoplates in two sections: (i) a cathodal 30 x 80 x 1.5 mm agarose strip (1%) without antibody and (ii) a 50 x 80 x 1.5 mm area containing 300 µl of rabbit antiserum. Buffers and other conditions are as described in (6).

Membrane components of normal monkey erythrocytes are identified, using rabbit anti-NMEM serum, in membranes from normal and parasitized cells and purified parasite (as a measure for a possible contamination by host cell membrane). Analyses of normal and infected membrane and purified parasite are shown in Fig. 7 A-C. Normal erythrocyte membranes (Fig. 7A) reveal three complex immune precipitates, component 1 at pI 6.0, component 2 and 3 at pI 5.8 and pI 5.5, respectively. Using the same amount of antigen (as determined by total amount of protein) components 2 and 3 are reduced to about half the concentration in membranes of infected cells and component 3 to more than two-fold. Component 1, possibly spectrin, is deleted (Fig. 7B). As documented in Fig. 7C, not more than 5% of erythrocyte membrane components 2 and 3 (focusing at lower pI's) are present in the parasite preparation. This indicates that less than 5% of the parasite protein constitutes erythrocyte membrane material.

Bidimensional isoelectric focusing-immune electrophoresis using our monkey serum has not yielded any immune precipitates even though these are very strong in simple crossed immune electrophoresis. This appears to be due to traces of urea remaining after isoelectric focusing.

2.3.3. Conclusions

Comparative analyses of membrane proteins from normal and parasitized erythrocytes and purified parasite indicate that there is only minimal cross-contamination when infected cells are subfractionated. Nevertheless, employing these high resolution techniques even minor contaminants have to be eliminated using methods outlined in section 1.3.

3. Protease treatment of normal monkey erythrocytes

Protease treatment (TPCK-trypsin or pronase) has proven very useful to explore the structure of membrane proteins in erythrocytes (7, 8) and lymphocytes (9).

In order to find optimal conditions for protease cleavage of infected cells, normal monkey erythrocytes are exposed to TPCK-trypsin (0.2 mg/ml) and pronase (0.05 mg/ml) at 37°C for 3 hr and 90 min, respectively. Using intact washed erythrocytes pronase selectively cleaves band 3, (mol. wt. ∿95,000 D), presumed the equivalent of band 3 of human erythrocytes, the major intrinsic membrane protein, into a 60,000 and 35,000D polypeptide fragment (P1 and P2 in Fig. 8). Trypsin treatment yields less homogeneous polypeptides with mol. wts. ∿65,000D. 50,000D and ∿40,000D. The cleavage of band 3 by trypsin is incomplete. Spectrin is not degraded by either enzyme.

Because band 3 has transport functions in human erythrocytes, it will be important to analyze whether modifications of band 3 in membranes from parasitized monkey erythrocytes account for altered transport characteristics described (10). This may adequately be explained by selective proteolytic cleavage of band 3.

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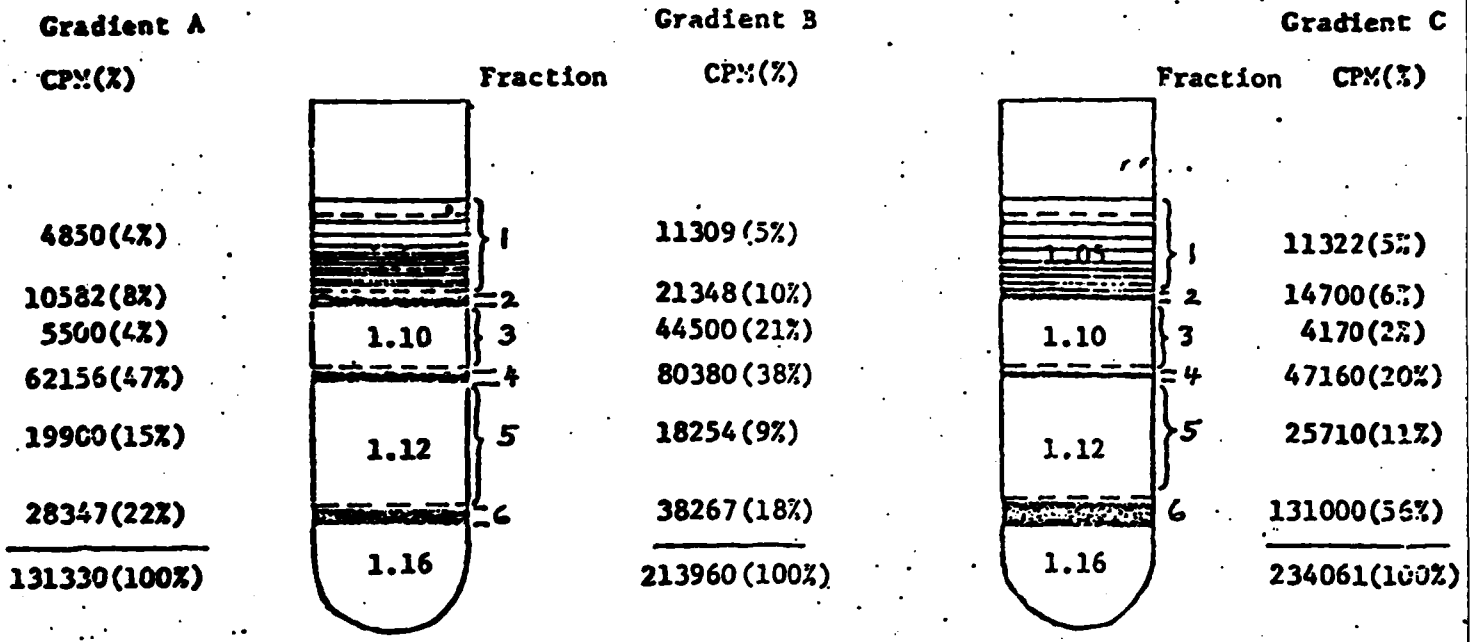


Figure 1: Subfractionation of purified intraerythrocytic P. knowlesi schizonts. The parasites were disrupted by nitrogen decompression (450 psi, 20 min; $2 \cdot 10^7$ parasites/ml in $NaCl/PO_4$ (0.13M/0.02M), pH 7.5). The homogenate is layered atop dextran step gradients (Dextran 170,000D dissolved in $NaCl/PO_4$, (0.13M/0.02M), pH 7.5; 1.05, 1.10, 1.12 and 1.16 density steps). Six fractions were collected from each gradient. For each gradient cpm and the 1% cpm in each fraction are given.

Gradient A: Parasite from erythrocytes, surface-labeled with $^{125}I$, using lactoperoxidase-catalyzed radioiodination. Fractionation of disrupted parasite.

Gradient B: Purified parasite isolated from radioiodinated erythrocytes as in A, and then surface-labeled by lactoperoxidase catalyzed radioiodination. Fractionation of disrupted parasite.

Gradient C: As gradient B, parasite not disrupted.

**Figure 2:** Crossed immune electrophoresis of purified membranes from monkey erythrocytes infected with *P. knowlesi*. Membrane proteins (150 µg; solubilized in 1% Triton X-100) were first separated electrophoretically in agarose/Triton and then electrophoresed at right angles into agarose containing monkey anti-parasite serum (0.12 ml/ 1 ml of agarose). Immune precipitates are numbered from 1 to 7 according to electrophoretic mobility in the first dimension. Coomassie blue staining.

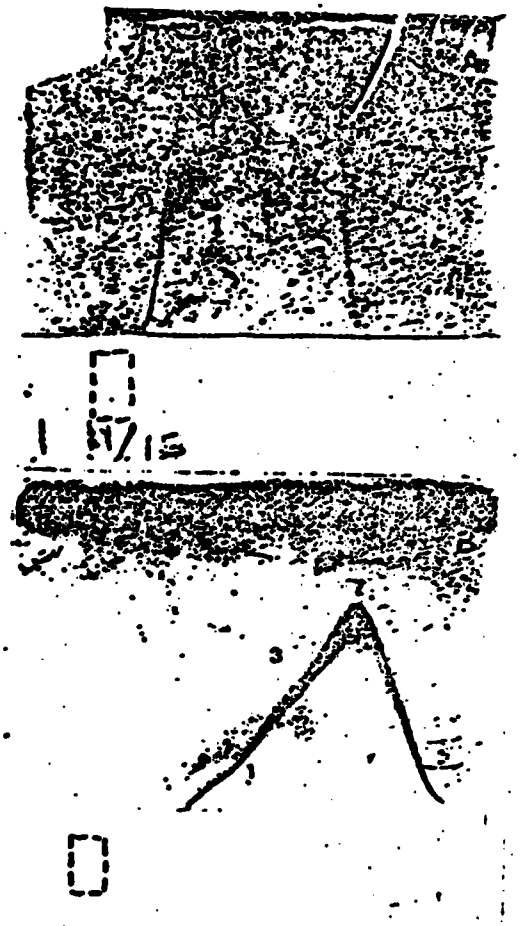

**Figure 3:** Crossed immune electrophoresis of purified *P. knowlesi* schizonts. Triton-solubilized parasites (150 µg protein, plate A and 75 µg protein, plate B) were separated electrophoretically in the first dimension and electrophoresed into agarose at right angles containing monkey anti-parasite serum (0.12 ml/1 ml agarose, plate A; 0.18 ml/1 ml agarose, plate 3). Other as in Figure 2.

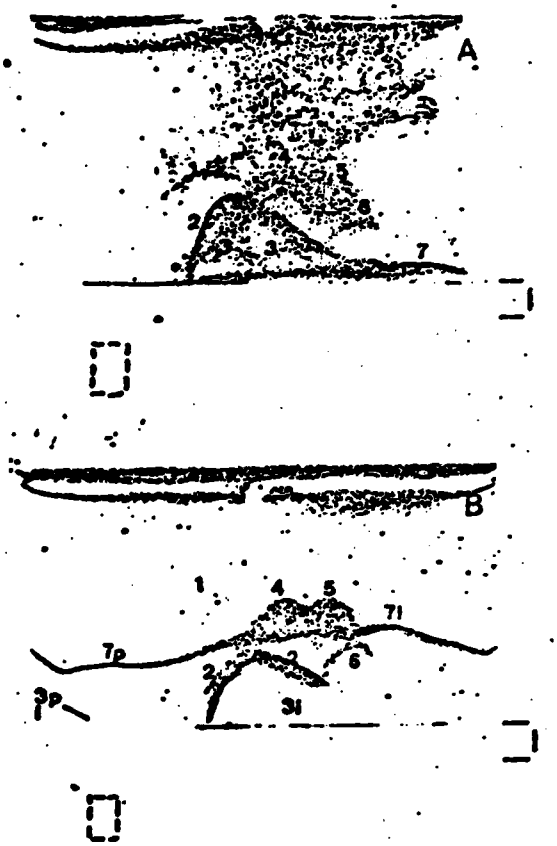


Figure 4: Crossed line immune electrophoresis of membranes purified from monkey erythrocytes injected with P. knowlesi (as in Fig. 1) but using an intermediate strip, I, containing normal erythrocyte membrane proteins (400 µg; plate A) and parasite (400 µg; plate B). Rest as in Figure 1.

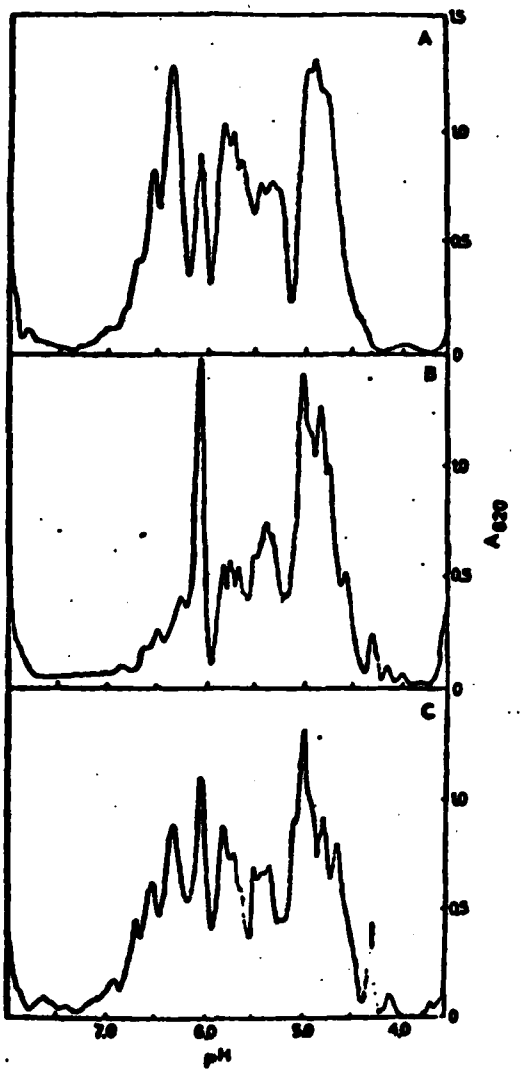


Figure 5: Isoelectric focusing of erythrocyte membranes of normal (panel A), parasite-infected cells (panel B) and purified parasites (panel C). Triton X-100 solubilized proteins (120 µg) are focused in 4% acrylamide/bisacrylamide containing 1% Triton, 8M urea and 2% ampholytes, pH 3.5-10.0. The abscissa gives the pH gradients and ordinates the absorbance for Coomassie blue staining at 620 min.

Figure 6

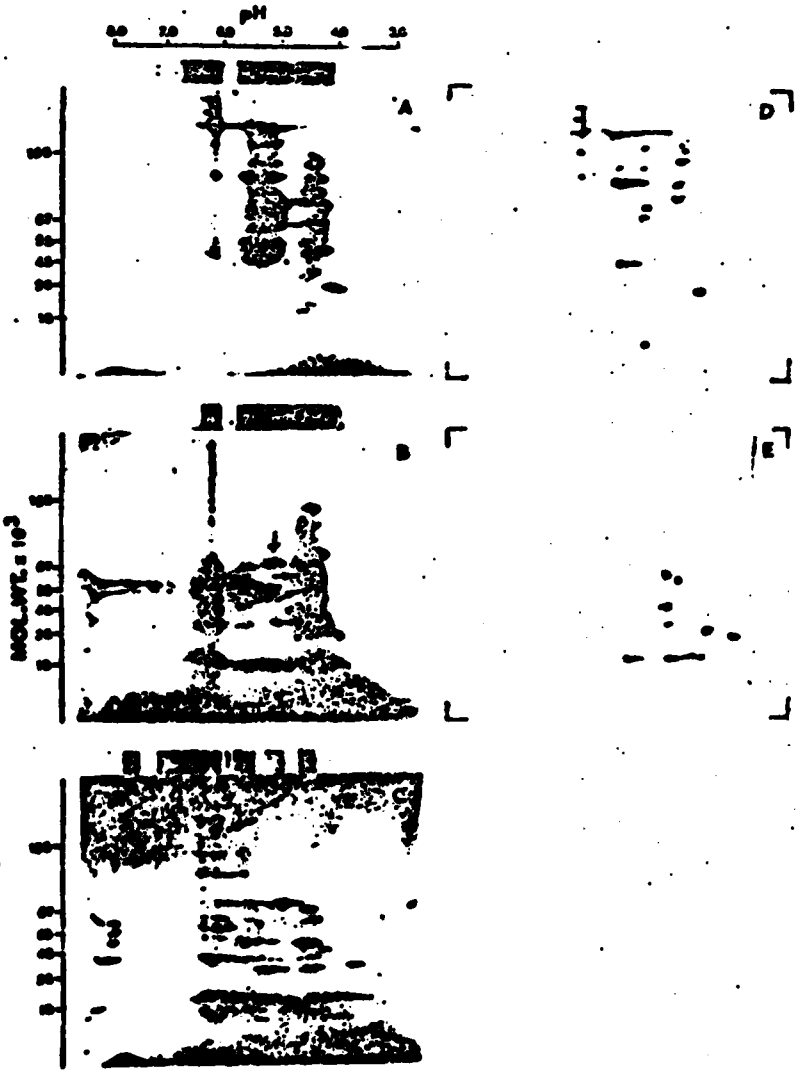

**Figure 6:** Bidimensional isoelectric focusing - dodecyl sulfate polyacrylamide gel electrophoresis (IEF-DS-PAGE) of

A. membranes of normal monkey erythrocytes

B. membranes of erythrocytes parasitized by P. knowlesi.

C. purified parasites.

The abscissa and the ordinate give the pH gradient and the molecular weights, respectively. 350-400 µg protein, solubilized in 1% Triton X-100, was focused in 4% acrylamide-bisacrylamide, containing 2% ampholytes (Ampholine 3.5-10.0), 8M urea, 1% Triton X-100. Prior to DS-PAGE the focused proteins are equilibrated with DS and dithiothreitol in the gel and positioned atop the acrylamide gradient gel (4-30%, Pharmacia, Sweden) for DS-PAGE in the second dimension. Coomassie blue protein staining.

D. Depiction of proteins in monkey erythrocyte membranes deleted or diminished in membranes of parasitized cells.

E. Protein components common to membranes of parasitized and the purified parasite.

Proteins unique to membranes of parasitized cells are marked with an arrow in B.

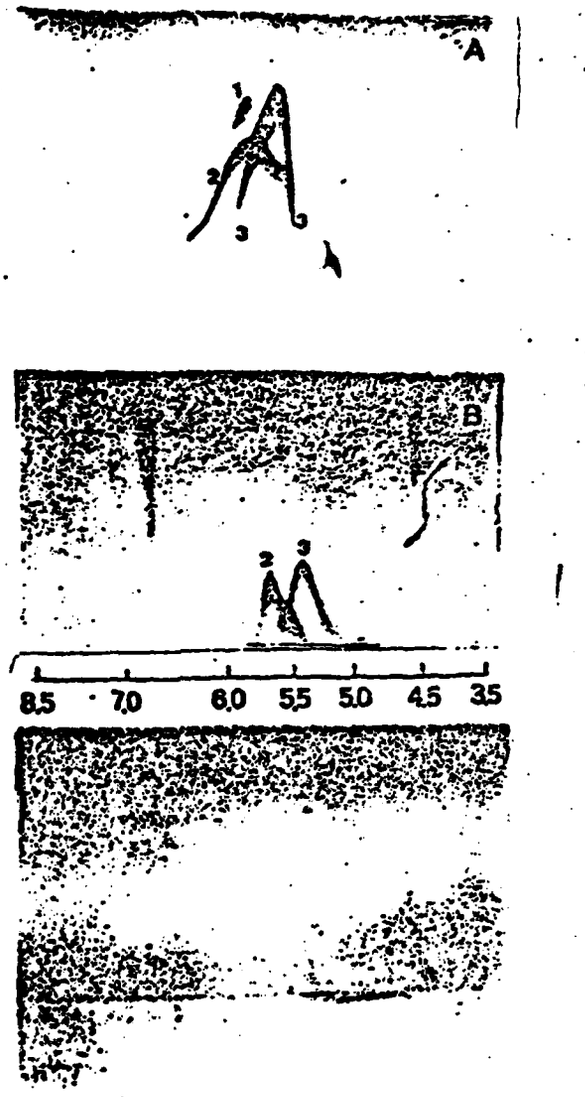


**Figure 7:** Bidimensional isoelectric focusing-immune electrophoresis of membrane proteins from normal erythrocytes (A), parasitized erythrocytes (B) and parasite (C). In all three experiments 350 µg of protein was focused in polyacrylamide-Triton/urea/ampholytes, pH 3.5-10.0 (4%/1%/8M/2%) and then electrophoresed into rabbit anti-NMEM serum (67 µl/1 ml agarose). The pH-gradient common for plate A-C is shown in plate B. The immune precipitates are numbered starting with 1 from the alkaline end of the pH gradient. Coomassie blue stain.

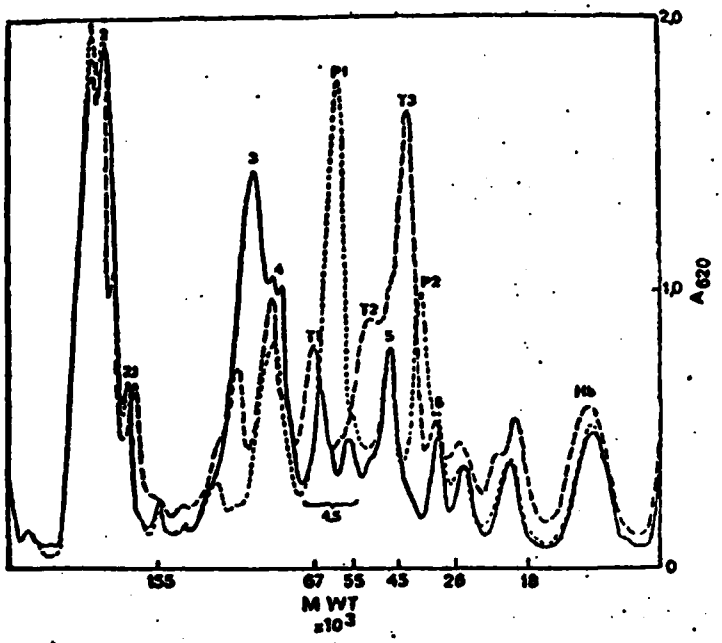


Figure 8: Protease treatment of normal monkey erythrocytes. Analysis of membrane proteins by DS-PAGE. Proteins are conventionally labeled as components 1-6 and Hb (hemoglobin). Washed intact erythrocytes were incubated with TPCK-trypsin 0.2 mg/ml, for 3 hr at 37°; broken line) or with pronase 0.05 mg/ml, for 90 min at 37°C; dotted line). Cleavage products of trypsin are designated as T1-T3 and of pronase as P1 and P2. The abscissa gives the molecular weights, the ordinate the absorbance for Coomassie brilliant blue at 620 nm.

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